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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

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Method for genomic typing of the Minor Histocompatibility Antigen HA-1

BACKGROUND OF THE INVENTION

Bone Marrow Transplantation (BMT) is the present treatment for hematological malignancies. One of the major drawbacks of allogeneic BMT is Graft versus Host Disease (GvHD). In recipients of allogeneic HLA genotypically identical BMT, GvHD occurs in 15-35% of all cases, depending on the age of the recipient and the amount of T-cell depletion of the graft. The development of GvHD in recipients of HLA identical BM grafts reflects the role of mHag in BMT. Disparities for mHag between BM donor and recipient can evoke strong Major Histocompatibility Complex (MHC) restricted cytotoxic and proliferative T-cells responses, which can be measured in vitro (Goulmy 1997). Human mHags showed variable phenotype frequencies in the population and they are inherited in a Mendelian fashion independent of HLA (reviewed in Goulmy 1997). Recently, the chemical nature of the first series of murine and human mHags were identified (Goulmy 1996). It became clear that mHags are naturally processed fragments of intracellular proteins that associate with MHC molecules. With regard to the human mHags two H-Y mHags were identified as peptides from the SMCY protein (Wang 1995, Meadows 1997), the non-Y linked HA-2 antigen consists of nine residues and is most probably encoded by an as yet unidentified member of the non-filament forming class I myosin family (den Haan 1995). The human genes encoding mHags- with exception of the gene encoding the male specific mHags SMCY-have not been identified, yet. With the identification of HA-1, the first human autosomal mH gene was recognized (den Haan 1998). The mHag HA-1 was found to be a nonapeptide with the amino acid residues VLHDDLLEA, derived from an allele of the partially sequenced KIAA0223 cDNA (GENBANK Acc. No. D86976). The HA-1 allelic counterpart found in the data base, VLRDDLLEA, differs in one amino acid , i.e. R, from HA-1 antigen (den Haan 1998). Based on the KIAA0223 cDNA information, we subsequently designed HA-1 allele specific primers for RT-PCR. The amplification products correlated exactly with the HA-1 phenotype determined by HA-1 specific cytotoxic T-cells. Exploring the allele specific primers on genomic DNA however failed. Isolation and sequencing of cosmid DNA encoding the HA-1 sequence identified an intron present in the middle of the HA-1 coding sequence.

gtg aga gcc acg ggg aca ccg agg cct ggg tgg aag aca gag cca gac cca agg gag gat gga ggg agg gac ttg ggg agg ctc aga agg gag gga ggc tca gat ggc agg gag ggc tgt gtg gaa gag gcc atg aca gct aag gct ctg agg gat gtg tag gag ttt ggt ggg gga gtc cct gag cgt aca ctg gct caa gag ggt gcc cac ttt att ttt ttt aaa gga tct gat ggc aat tag gag gga aag gca gag gaa atg tcc cat gca cag gct cag aaa cac gga aac aga gaa tgc att tgg ggg cca agg tgt ggg gtg ccg ctg gtg tag gat gaa ggc atg aca acg cca ggc aga agg gca at SEQ ID NO 1

This sequence represents part of the interrupting intron (indicated as intron a in figure 1), the first nucleotide of this sequence being the first nucleotide of intron a. The present invention thus also relates to an isolated polynucleic acid identified by SEQ ID NO 1, or an isolated polynucleic acid displaying at least 80%, or at least 90%, or at least 95%, or at least 99% sequence homology to SEQ ID NO 1, or any fragment of said polynucleic acids that can be used as a primer or as a probe.

Sequence information corresponding to another part of intron a, more particularly the part which is situated in front of exon b (figure 1) has been disclosed in the EMBL database under accession number AC004151. However, this sequence is not suitable for the design of primers for the above-mentioned method, since the length of the amplified fragment would lower the efficacy of the amplification reaction.

For detection of the amplification product mentioned in step b above, different methods known in the art, may be used. One method consists of subjecting the mixture obtained after the amplification reaction to gel electrophoresis and visually detecting the amplification product after nucleic acid staining. Alternatively, the amplification product may be labeled, for instance by using labeled primers, and may be captured on a solid support, for instance by hybridization, and may be detected on the solid support. It is clear, however, that other detection methods are also within the scope of the present invention.

According to a more preferred embodiment, the present invention relates to a method as indicated above, further characterized in that:

said at least one pair of primers comprises a 5'-primer that specifically hybridizes to a target region comprising the nucleotides at position 4 or at positions 4 and 8 in the HA-1 allele, or

said at least one pair of primers comprises a 3'-primer that specifically hybridizes to a

a. The target region of the 3'-primers comprises the polymorphic nucleotides at positions 4 and 8 in the HA-1 coding sequence (figure 1) and partly overlaps with the sequence identified by SEQ ID NO 1. Set 2 consists of a common 3'-primer and two different 5'-primers. The target region of the common primer is located in the sequence identified by SEQ ID NO 1, whereas the target regions of the 5'-primers are located in exon a and comprise the polymorphic nucleotides at positions 4 and 8. Example 3 shows a genomic typing experiment making use of these primer sets.

According to another preferred embodiment, the present invention relates to a diagnostic kit for genomic typing of alleles of the Minor Histocompatibility Antigen HA-1 according to any of the methods indicated above, with said kit comprising:

a) at least one primer according to any of the methods indicated above;

b) optionally, an enzyme and/or reagents enabling the amplification reaction;

c) optionally, means enabling detection of the amplified products.

According to another preferred embodiment, the present invention relates to a method for genomic typing of alleles of the Minor Histocompatibility Antigen HA-1 in a sample, with said method comprising:

a) amplifying a fragment of said alleles, with said fragment comprising at least one polymorphic nucleotide, by use of at least one pair of primers specifically hybridizing to conserved target regions in said alleles;

b) hybridizing the amplified product of step a) to at least one probe specifically hybridizing to a target region comprising one or more polymorphic nucleotides in said allele;

c) inferring from the result of step b) which HA-1 allele is present in said sample.

According to a more peferred embodiment, the present invention relates to a method as indicated above, further characterized in that said alleles of the Minor Histocompatibility Antigen HA-1 are the H allele and the R allele.

According to an even more preferred embodiment, the present invention relates to a method as indicated above, further characterized in that said at least one pair of primers comprises a 5'-primer specifically hybridizing to a conserved target region in exon a and/or a 3'-primer specifically hybridizing to a conserved target region in intron a, with exon a and intron a being indicated in figure 1.

Ideally, the target region of said 3'-primer is located in the sequence identified as SEQ ID NO 1.

to target regions overlapping with the exon a-intron a boundary. The probes with SEQ ID NO 11 to 16 have been optimized to function in combination at the same conditions in a LiPA assay (see below). The skilled man will recognize that the probes and primers with SEQ ID NO 2 to 16 may be adapted by addition or deletion of one or more nucleotides at their extremities. Such adaptations may be required if the conditions of amplification or hybridization are changed, or if the amplified material is RNA instead of DNA, as is the case in the NASBA system. Different techniques can be applied to perform the sequence-specific hybridization methods of the present invention. These techniques may comprise immobilizing the amplified HA-1 polynucleic acids on a solid support and performing hybridization with labelled oligonucleotide probes. Genomic polynucleic acids may also be immobilized on a solid support without prior amplification and subjected to hybridization. Alternatively, the probes may be immobilized on a solid support and hybridization may be performed with labelled HA-1 polynucleic acids, preferably after amplification. This technique is called reverse hybridization. A convenient reverse hybridization technique is the line probe assay (LiPA). This assay uses oligonucleotide probes immobilized as parallel lines on a solid support strip (Stuyver et al., 1993). It is to be understood that any other technique for genomic typing of HA-1 alleles is also covered by the present invention.

It is clear that the present invention also relates to any of the primers with SEQ ID NO 2 to 10 and to any of the probes with SEQ ID NO 11 to 16, with said primers and said probes being for use in a method for genomic typing of alleles of the Minor Histocompatibility Antigen HA-1.

According to another preferred embodiment, the present invention relates to a diagnostic kit for genomic typing of alleles of the Minor Histocompatibility Antigen HA-1 according to any of the sequence-specific hybridization methods indicated above, with said kit comprising:

a) at least one primer according to any of the methods indicated above;

b) at least one probe according to any of the methods indicated above;

c) optionally, an enzyme and/or reagents enabling the amplification reaction, and/or reagents enabling the hybridization reaction.

DEFINITIONS

The following definitions and explanations will permit a better understanding of the present

"Specific hybridization" of a probe to a target region of the HA-1 polynucleic acids means that said probe forms a duplex with part of this region or with the entire region under the experimental conditions used, and that under those conditions said probe does not form a duplex with other regions of the polynucleic acids present in the sample to be analysed.

"Specific hybridization" of a primer to a target region of the HA-1 polynucleic acids means that, during the amplification step, said primer forms a duplex with part of this region or with the entire region under the experimental conditions used, and that under those conditions said primer does not form a duplex with other regions of the polynucleic acids present in the sample to be analysed. It is to be understood that "duplex" as used hereby, means a duplex that will lead to specific amplification.

"Specific amplification" of a fragment of the HA-1 polynucleic acids means amplification of the fragment for which the primers were designed, and not of any other fragment of the polynucleic acids present in a sample.

The fact that amplification primers do not have to match exactly with the corresponding target sequence in the template to warrant proper amplification is amply documented in the literature (Kwok et al., 1990). However, when the primers are not completely complementary to their target sequence, it should be taken into account that the amplified fragments will have the sequence of the primers and not of the target sequence. Primers may be labelled with a label of choice (e.g. biotine). The amplification method used can be either polymerase chain reaction (PCR; Saiki et al., 1988), ligase chain reaction (LCR; Landgren et al., 1988; Wu & Wallace, 1989; Barany, 1991), nucleic acid sequence-based amplification (NASBA; Guatelli et al., 1990; Compton, 1991), transcription-based amplification system (TAS; Kwoh et al., 1989), strand displacement amplification (SDA; Duck, 1990) or amplification by means of Qß replicase (Lomeli et al., 1989) or any other suitable method to amplify nucleic acid molecules known in the art.

Probe and primer sequences are represented throughout the specification as single stranded DNA oligonucleotides from the 5' to the 3' end. It is obvious to the man skilled in the art that any of the below-specified probes can be used as such, or in their complementary form, or in their RNA form (wherein T is replaced by U).

The probes according to the invention can be prepared by cloning of recombinant plasmids containing inserts including the corresponding nucleotide sequences, if need be by excision of the latter from the cloned plasmids by use of the adequate nucleases and recovering them, e.g. by

exact sensitivity and specificity of a particular probe, whether perfectly complementary to its target or not. The importance and effect of various assay conditions are explained further herein.

**The stability of the [probe : target] nucleic acid hybrid should be chosen to be compatible with the assay conditions. This may be accomplished by avoiding long AT-rich sequences, by terminating the hybrids with G:C base pairs, and by designing the probe with an appropriate Tm. The beginning and end points of the probe should be chosen so that the length and %GC result in a Tm about 2-10°C higher than the temperature at which the final assay will be performed. The base composition of the probe is significant because G-C base pairs exhibit greater thermal stability as compared to A-T base pairs due to additional hydrogen bonding. Thus, hybridization involving complementary nucleic acids of higher G-C content will be more stable at higher temperatures.

**Conditions such as ionic strength and incubation temperature under which a probe will be used should also be taken into account when designing a probe. It is known that the degree of hybridization will increase as the ionic strength of the reaction mixture increases, and that the thermal stability of the hybrids will increase with increasing ionic strength. On the other hand, chemical reagents, such as formamide, urea, DMSO and alcohols, which disrupt hydrogen bonds, will increase the stringency of hybridization. Destabilization of the hydrogen bonds by such reagents can greatly reduce the Tm. In general, optimal hybridization for synthetic oligonucleotide probes of about 10-50 bases in length occurs approximately 5°C below the melting temperature for a given duplex. Incubation at temperatures below the optimum may allow mismatched base sequences to hybridize and can therefore result in reduced specificity.

**It is desirable to have probes which hybridize only under conditions of high stringency. Under high stringency conditions only highly complementary nucleic acid hybrids will form; hybrids without a sufficient degree of complementarity will not form. Accordingly, the stringency of the assay conditions determines the amount of complementarity needed between two nucleic acid strands forming a hybrid. The degree of stringency is chosen such as to maximize the difference in stability between the hybrid formed with the target and the non-target nucleic acid.

**Regions in the target DNA or RNA which are known to form strong internal structures inhibitory to hybridization are less preferred. Likewise, probes with extensive self-complementarity should be avoided. As explained above, hybridization is the association of two single strands of complementary nucleic acids to form a hydrogen bonded double strand. It is

Table 3

Cellular and genomic typing for HA-1 in three HLA-A*0201 positive families.

Figure 1

Sequences and genomic structure of the HA-1 locus. Figure 1a, coding sequences of the H and R alleles of HA-1. Bold characters indicate the polymorphic nucleotides. Figure 1b, exon-intron boundaries of the HA-1 locus. Exon sequences are shown in uppercase, intron sequences in lowercase.

Figure 2

Genomic typing of HA-1 alleles in clinical samples. Genomic typing was performed by sequence-specific amplification, by use of the two primer sets of Table 1. The two upper fragments in the gel originate from the H-allele, the two lower fragments from the R-allele.

EXAMPLES

Example 1. Materials and Methods

Cell culture and isolation of genomic DNA:

Genomic DNA was isolated from frozen peripheral blood lymphocytes (PBL) with the High Pure Template Purification Kit from Boehringer Mannheim according to the manufacturers description. EBV-transformed LCLs cells were cultured in RPMI-1640 containing 3mM L-glutamine and 10% FCS. For DNA isolation the cells were harvested, washed twice with phosphate buffered saline (PBS), resuspended in 200 µl and kept at -20C until use. For each DNA isolation $2x10^6$ cells were used.

Genomic PCR:

For each PCR reaction 100-200 ng of genomic DNA were used. Amplifications were performed with 20 pmol of each primer in 100 µl of 10mM Tris/HCl (pH8.4) buffer, containing 50mM KCl, 4mM MgCl2, 0.06mg/ml BSA, 0.5 mM dNTP's and 2.5 units Taq polymerase (Roche Molecular Systems, Brancheburg, New Jersey). All reactions started with a denaturation step of 5 min. at

Example 3 Allele-specific PCR on genomic DNA

For the genomic allele-specific typing two different primer sets were designed. Both sets do contain a common primer and one specific for either the HA-1 H-allele or R-allele (table 1). The common primer of set 1 is derived from the exon encoding the first four amino acids of the HA-1 peptide. The H/R primers contain intronic sequences , the splice donor site and the allele specific part of the exon sequence. Set 2 consists of a common primer derived from the intron identified in pTCF-HA-1 and exon derived primers covering the H- and R-allele. Amplification with primer set 1 resulted in a 190bp fragment, primer set 2 gave a 331bp fragment. Both primer sets showed the expected length of fragments and are suitable for genomic typing. Because the primers were chosen in such a way, that they should amplify the DNA under identical PCR conditions, a combination of both primer sets can be used in the same PCR reaction. In this case, a third fragment of 535bp was observed due the amplification of the DNA between the two different common primers (data not shown).

Example 4 Family studies

The feasibility of genomic typing was carried out on 24 members belonging to three HLA-A*0201 positive families. The results of the DNA typing was compared with the mHag HA-1 CTL typing (table 3) and showed an exact correlation. Figure 2 shows the genomic DNA analysis of the HA-1 locus in a representative family. The bone marrow donor (06) and recipient (02) were HLA-identical. The donor was homozygous for the R-allele. The recipient was heterozygous (H/R) and therefore presenting the HA-1 antigen at the cell surface. This mismatch resulted in GvHD, thus the T-cells of the donor reacted against the mHag of the recipient. In this familiy the donor and recipient were HLA-identical, but they had a mismatch in the HA-1 sequence. The same disparities for HA-1 could be observed in family 2. Again the donor (07) was homozygous for the R-allel and the patient (02) was heterozygous (H/R), resulting in GvHD. Family 3 represents the segregation of the HA-1 H-allele in three generations in an healthy family. The H-allele is derived from the grandfather (01) and is inherited by two generations. The grandmother (00) although HLA-A*0201 positive is homozygous for the R-allele. Their children (03, 04, and 05) are all heterozygous for the HA-1 locus. The child 04 married individual 34 who is HLA-

34	-	R/R
82	-	R/R
83	-	R/R
84	+	H/R
85	-	R/R

Example 5 Typing of HA-1 alleles by the LiPA method

The following method for typing of the HA-1 alleles H and R in a sample, is based on the LiPA technology (Stuyver et al, 1993). For each PCR reaction 100-200 ng of genomic DNA is used. Amplifications are performed with 20 pmol of each primer in 100 µl of 10mM Tris/HCl (pH8.4) buffer, containing 50mM KCl, 4mM MgCl2, 0.06mg/ml BSA, 0.5 mM dNTP's and 2.5 units Taq polymerase (Roche Molecular Systems, Brancheburg, New Jersey). All reactions start with a denaturation step of 5 min. at 95°C. The cycling conditions for all primer combinations are 95°C for 1 min. and 65°C for 1 min. for ten cycles. Followed by 20 cycles at 95°C for 1 min., 62°C for 1 min. , 72°C for 1 min., and an extension of the last step for 5 min. at 72°C. The HA-1 alleles are subsequently typed by a reverse hybridization step to oligonucleotide probes that are immobilized on a nitro-cellulose strip. Probes specifically hybridizing to the R-allele are for instance HA1-R1(1) (SEQ ID NO 11), HA1-R1(2) (SEQ ID NO 12) and HA1-R1(3) (SEQ ID NO 13). Probes specifically hybridizing to the H-allele are for instance HA1-H1(1) (SEQ ID NO 14), HA1-H1(2) (SEQ ID NO 15) and HA1-H1(3) (SEQ ID NO 16). The hybridization is performed in 5x SSPE, 0.5% SDS at 56°C for 30 min. A stringent washing step is carried out in 2x SSPE, 0.1% SDS at 56°C for 10 min.

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Santamaria P., Boyce J.M., Lindstrom A.L. *et al.* HLA class II "typing" : direct sequencing of

CLAIMS

1. Method for genomic typing of alleles of the Minor Histocompatibility Antigen HA-1 in a sample, with said method comprising:

a) contacting the genomic polynucleic acids in the sample with at least one pair of primers, whereby the 5'- and/or the 3'-primer of said at least one pair of primers specifically hybridize to target regions comprising polymorphic nucleotides in said alleles, and performing an amplification reaction;

b) for each of said at least one pair of primers detecting whether or not in step a) an amplification product is formed;

c) inferring from the result of step b) which HA-1 allele is present in said sample.

2. Method according to claim 1, further characterized in that said alleles of the Minor Histocompatibility Antigen HA-1 are the H allele and the R allele.

3. Method according to any of claims 1 and 2, further characterized in that:

said at least one pair of primers comprises a 5'-primer that specifically hybridizes to a target region comprising the nucleotides at position 4 or at positions 4 and 8 in the HA-1 allele, or

said at least one pair of primers comprises a 3'-primer that specifically hybridizes to a target region comprising the nucleotides at position 8 or at positions 4 and 8 in the HA-1 allele, with said positions being indicated in figure 1.

4. Method according to claim 3, further characterized in that:

said 5'-primer is combined with a 3'-primer specifically hybridizing to a target region in intron a, and/or

said 3'-primer is combined with a 5'-primer specifically hybridizing to a target region in exon a,

with intron a and exon a being indicated in figure 1.

5. Method according to any of claims 1 to 4, further characterized in that the primers are

11. A primer for use in a method according to any of claims 1 to 10 for genomic typing of alleles of the Minor Histocompatibility Antigen HA-1.

12. A probe for use in a method according to any of claims 6 to 10 for genomic typing of alleles of the Minor Histocompatibility Antigen HA-1.

13. An isolated polynucleic acid identified by SEQ ID NO 1, or an isolated polynucleic acid displaying at least 80% sequence homology to SEQ ID NO 1, or any fragment of said polynucleic acids that can be used as a primer or as a probe.

14. A diagnostic kit for genomic typing of alleles of the Minor Histocompatibility Antigen HA-1 according to any of claims 1 to 5, with said kit comprising:
a) at least one primer according to any of claims 1 to 5;
b) optionally, an enzyme and/or reagents enabling the amplification reaction;
c) optionally, means enabling detection of the amplified products.

15. A diagnostic kit for genomic typing of alleles of the Minor Histocompatibility Antigen HA-1 according to any of claims 6 to 10, with said kit comprising:
a) at least one primer according to any of claims 6 to 10;
b) at least one probe according to any of claims 6 to 10;
c) optionally, an enzyme and/or reagents enabling the amplification reaction, and/or reagents enabling the hybridization reaction.

ABSTRACT

The present invention provides a method for genomic typing of alleles of the Minor Histocompatibility Antigen HA-1 in a sample, with said method comprising: a) contacting the genomic polynucleic acids in the sample with at least one pair of primers, whereby the 5'- and/or the 3'-primer of said at least one pair of primers specifically hybridize to target regions comprising polymorphic nucleotides in said alleles, and performing an amplification reaction; b) for each of said at least one pair of primers detecting whether or not in step a) an amplification product is formed; c) inferring from the result of step b) which HA-1 allele is present in said sample. The present invention also provides a method for genomic typing of alleles of the Minor Histocompatibility Antigen HA-1 in a sample, with said method comprising: a) amplifying a fragment of said alleles, with said fragment comprising at least one polymorphic nucleotide, by use of at least one pair of primers specifically hybridizing to conserved target regions in said alleles; b) hybridizing the amplified product of step a) to at least one probe specifically hybridizing to a target region comprising one or more polymorphic nucleotides in said allele; c) inferring from the result of step b) wich HA-1 allele is present in said sample. In addition, the present invention provides primers and probes for use in the above-mentioned methods. Diagnostic kits enabling said methods are also provided.

Fig.1 :Sequences encoding the HA-1 antigen locus

A

HA-1 R-allele:

GTG TTG CGT GAC GAC CTC CTT GAG GCC

V L **R** D D L L E A

HA-1 H-allele:

GTG CTG CAT GAC GAC CTC CTT GAG GCC

V L **H** D D L L E A

B